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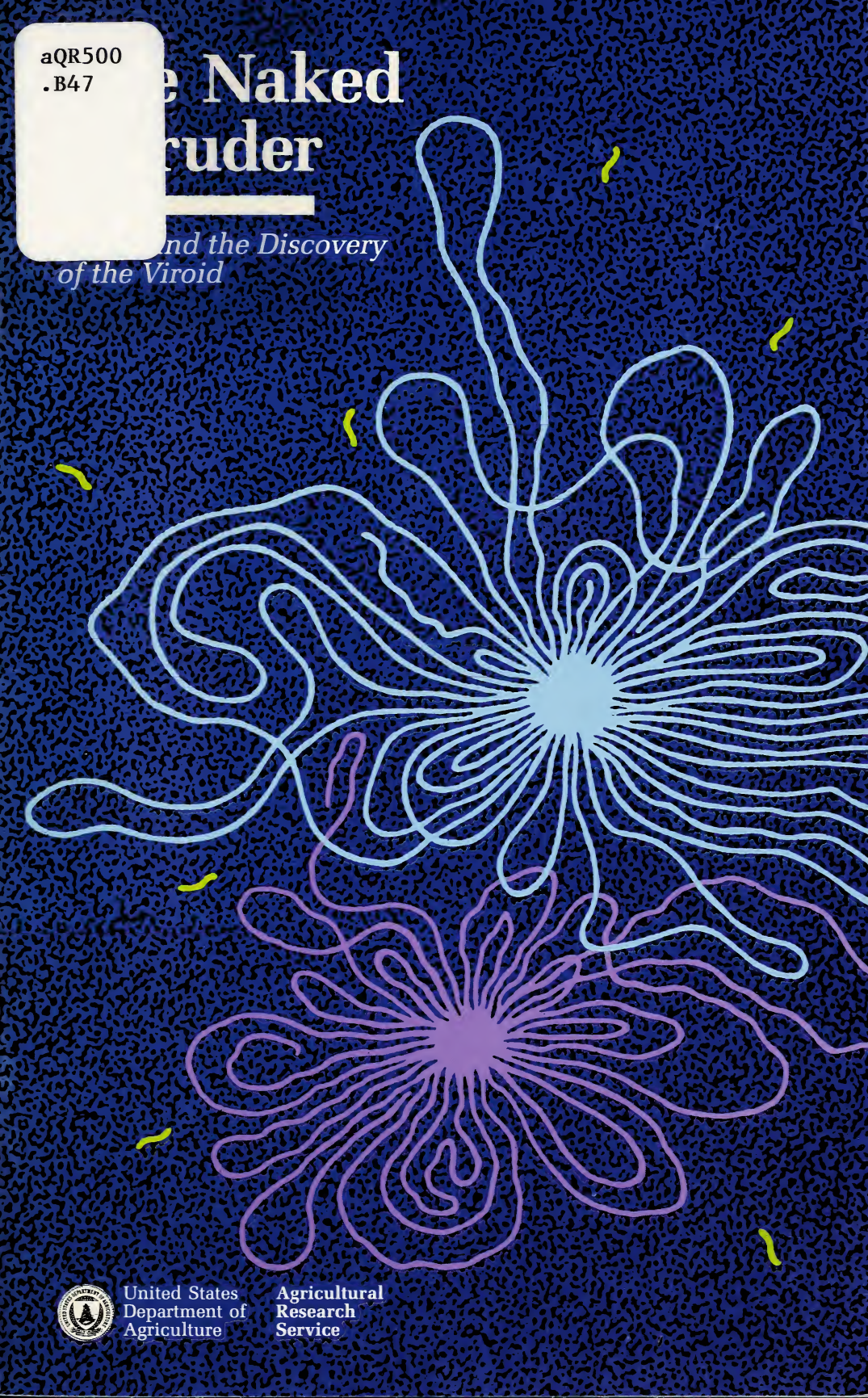

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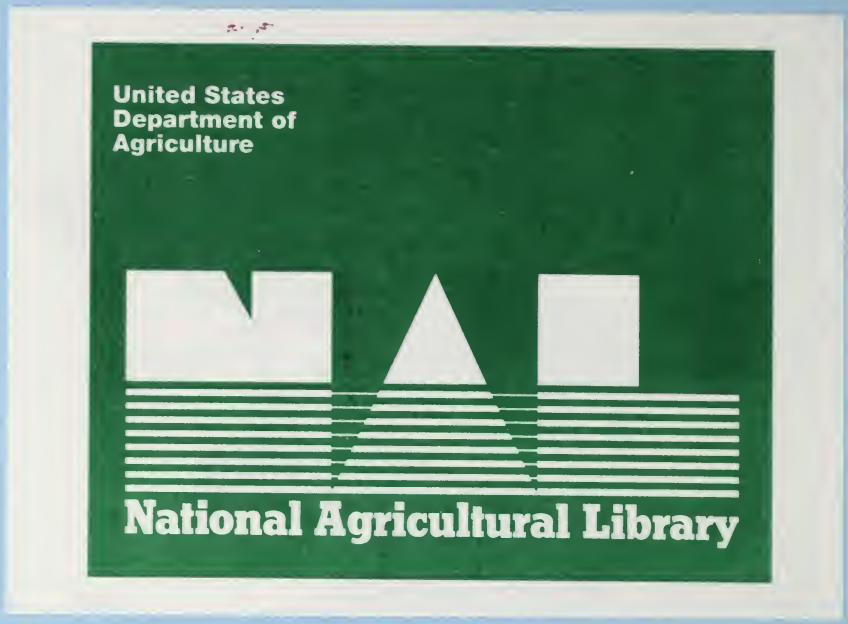

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Cover
The world's first known viroid is shown as yellow-green rods in artist's rendition of an electron microscope photograph. The small size of the viroid is apparent when compared with a viral DNA strand shown in light blue. (Cover by Roy Nash.)

The Naked Intruder

USDA and the Discovery of the Viroid

by Stephen M. Berberich

A virus is difficult to imagine. It doesn't grow by cell division as do living things. It has no metabolism. It can't move about by itself. But because of the deadly diseases viruses cause, scientists suspected their existence more than 50 years before they actually found one.

In 1939, 4 years after he was the first to show that a virus could be isolated, American biochemist Wendell Meredith Stanley said, "There is in reality a continuum from simple to complex structures, from molecules to organisms, and after all there is no great difference between the two." Stanley's comment now seems even more astute since the discovery of the viroid. But unlike finding the long-sought virus, discovery of the even simpler viroid was not supposed to happen.

Introduction

Viruses that cause such human diseases as AIDS, polio, or influenza get a lot of attention. Research on these frightening human pathogens is important to everyone. There are, however, other killer viral pathogens that are arguably just as important, though they aren't as well known. These pathogens don't make people sick, but they do infect the crops and livestock that provide our food.

For example, the notorious foot-and-mouth disease is caused by a virus. So are deadly rinderpest in cattle, avian influenza in poultry, African swine fever, and dozens of other diseases feared by livestock farmers worldwide. Even relatives of the human AIDS virus infect livestock—horses, pigs, cattle, and others—to disarm body defenses and eventually kill. Every crop can get at least 1 of 400 viruses. For example, yellow dwarf virus of barley, oats, wheat, and other cereals—a worldwide problem—cuts U.S. harvests alone by about $300 million a year.

Scientists developing vaccines against animal viruses, or breeding virus-resistant crops, are as skilled and informed as medical researchers. But their pioneering work gets far less public attention.

This is a story of one such agricultural scientist and his exemplary influence on virus studies. He works in a small U.S. Department of Agriculture (USDA) laboratory with a couple of

technicians at Beltsville, Maryland. Yet his discovery has profoundly affected how all virologists—crop, animal, and human—view their science and conduct their investigations worldwide.

Theodor O. Diener, with the USDA's Agricultural Research Service (ARS), made his discovery in 1971. It was a new kind of viruslike pathogen that he called the viroid. His isolation and identification of that first viroid, and his evidence that it causes the potato spindle tuber disease, are today likened to the discoveries of bacteria in the late 1800's and of viruses in the first half of the 20th century. The discovery of viroids has opened new avenues to the study of diseases of plants, animals, and humans.

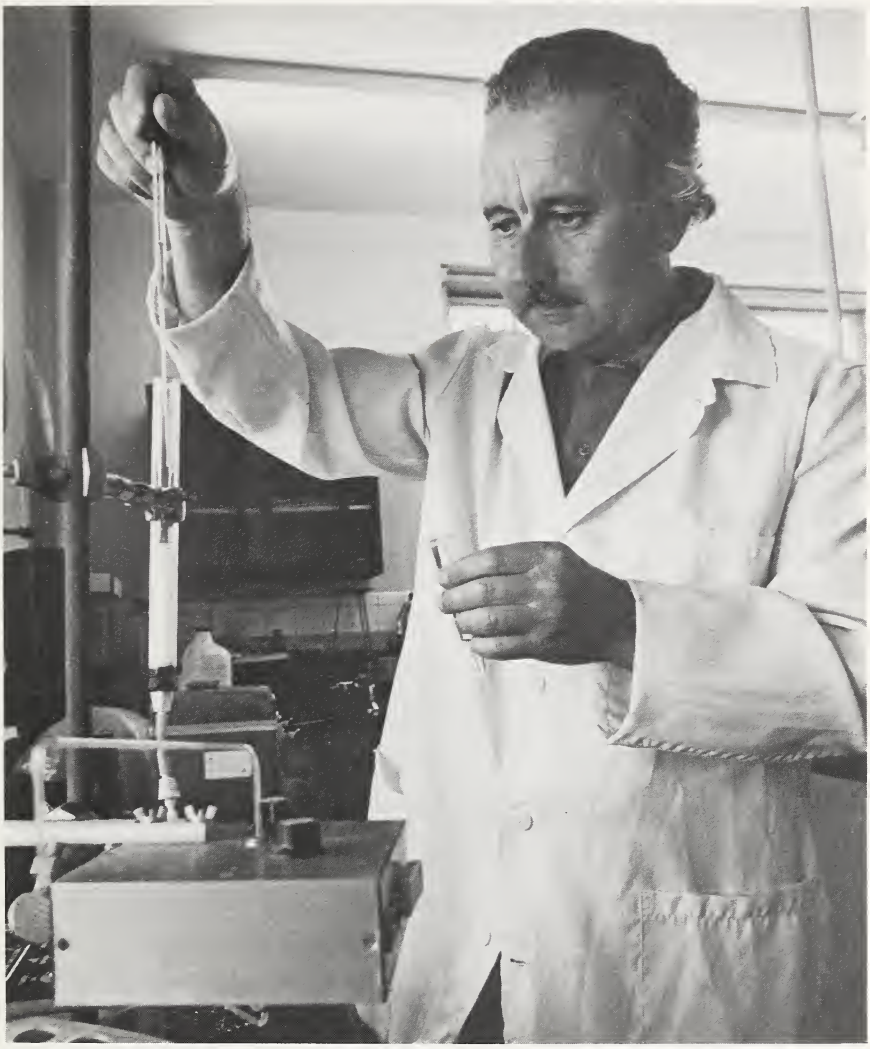
In 1987, Diener's insight and leadership in viroid research earned him both the International Wolf Foundation Prize in agriculture and the U.S. National Medal of Science. He is the pioneer, the father, of viroid research. At least 15 different plant diseases, which scientists had thought were caused by viruses, are now known to be caused by viroids. Many more, including some diseases affecting mammals, are also suspected to be viroid-incited.

The Elusive Potato Disease Agent

The story of the viroid discovery actually begins in 1961 with ARS plant pathologist William B. Raymer of the Potato Diseases Investigations Laboratory at the Beltsville Agricultural Research Center near Washington, D.C. Raymer introduced serological screening for viruses into the USDA's potato breeding program. That program, carried out in cooperation with various State agricultural experiment stations, has for the past three decades released most of the high-yielding, disease-resistant potato varieties in the United States.

Raymer made antisera in rabbits for different potato viruses. Each antiserum was used to detect a specific virus and keep it out of research fields and greenhouses. The mysterious cause of the potato spindle tuber disease was one of Raymer's targets.

Potato spindle tuber was quite an infamous disease among plant pathologists. Although they found little difficulty in transferring it from infected potato leaves to healthy plants, no one could ever isolate a virus. If it could be transmitted, they reasoned, it was some sort of infectious agent. Scientists had assumed for 50 years that the viruslike symptoms of the disease were in fact the dirty work of a virus.



Theodor O. Diener was new to Beltsville when William Raymer sought his help to find the agent for potato spindle disease. Available centrifuge techniques were inadequate to detect the disease agent.

No one, however, seemed overly concerned, recalls long-time USDA potato breeder/pathologist Raymon E. Webb, because the disease was not actually a major problem for potato farmers. It was the scare factor of the mysterious disease that brought it under ARS scrutiny, Webb says. "Maybe potato spindle could rear up and damage all our breeding potatoes one year, and we wouldn't be able to say why or do much about it."

The problem for Webb, Raymer, and other investigators was that the disease is not noticeable when it first infects potato plants. The symptoms aren't strong the first year. But planting

Growth stunting and leaf distortions in tomato plant on right are caused by potato spindle tuber viroid.

Small leaves from tomato plant are infected with potato spindle tuber disease.

infected seedpieces for the next crop leads to a second-year harvest of very spindly, twisted tubers.

It was known as "sort of a social disease," says Webb. "Nobody would ever admit that it was in their breeding lines, but you knew it was around. We assumed it was a virus. But insects wouldn't transmit it from plant to plant in test cages. We tried transmitting the disease with aphids, leafhoppers, flea beetles, and grasshoppers in Maine because our field plots were up there. But the transmission was too low and too slow for this to be a virus as we knew them."

Another false assumption made by some was that the disease agent was a member of the yellows group of viruses, specifically that it was the same as the yellows disease called purple top of potatoes. Raymer disagreed. He had studied purple top while at Oregon State University at Corvallis. After joining USDA, he benefited from working with a collection of potato diseases preserved in quarantined plants by the original USDA potato investigator E.S. Schultz at the ARS breeding station at Presque Isle, Maine. After Raymer inoculated test plants with spindle tuber disease samples from the Schultz collection (still used by the ARS breeding program today), he dispelled the idea that the disease is caused by a yellows virus.

With his curiosity aroused, Raymer's obvious strategy was to purify the agent and shoot it into rabbits to make an antiserum—a common diagnostic tool for many viruses. It didn't work. He had no way of knowing at the time that the viroid to blame contained no protein, which a rabbit's immune system needs to make an antiserum to the agent.

Another problem in studying the agent was that scientists couldn't collect enough of it. Because the disease took a full season or more to show itself, use of potato plants was not practical for assaying the agent of the infection. Severe symptoms developed only in the tubers, not in the leaves where the disease could be monitored more readily. It took a full season, or maybe two, for researchers to see if the tubers became spindly.

In 1962, Raymer and fellow ARS plant pathologist Muriel O'Brien at Beltsville came up with a breakthrough in convenience—a simple bioassay for the infectious agent. They found that the agent, whatever it was, was easily transmitted in tomato plants. A tomato plant became dramatically stunted within 2 weeks.

Now they could get lots of diseased leaves quickly. High-speed centrifugation, a standard method to purify viruses, would surely turn up the virus, thought Raymer and O'Brien. "Bottling" the virus, capturing it, seemed imminent.

They placed concentrated extracts of infected tomato leaves into a test tube and spun them in a high gravitational field. The centrifuge spins the relatively heavy virus particles into a clump or pellet.

The scientists recovered a pellet and rubbed it onto leaves of healthy tomato plants. They sat back, expecting a tremendous amount of infectivity, because they thought they had a very concentrated amount of the virus. Instead, nearly no disease symptoms developed.

They repeated the experiment. Even after spinning at 100,000 times the force of gravity for 4 hours, the resulting pellet caused no pronounced disease symptoms. This time, though, they also inoculated healthy tomato plants with some of the extract that didn't spin into the pellet—extract from the upper or lightweight part, the supernatant, of the test tube. Sure enough, the plants got sick. The strange infective agent was still there.

"People came to me and asked what kind of mistake could they be making," recalls Myron K. Brakke, an ARS chemist credited with developing centrifugation techniques for efficient purification of viruses, nucleic acids, proteins, and cellular protein factories called ribosomes. "People asked, 'Could the virus have fallen apart in the centrifuge?' Other people thought their tomato assay was wrong. Perhaps the greenhouse man was killing virus-carrying insects with his fingers and thereby infecting the plants. Raymer's team took all kinds of precautions to protect their tomato plants from contamination," says Brakke.

A Pioneering Lab

Raymer went to Theodor Diener, who was new to Beltsville but not unknown around the center.

Russell L. Steere, botanist and chief of the new Plant Virology Pioneering Laboratory, had hired Diener in 1959 from Washington State University where he had been an associate plant pathologist studying host-virus interactions on fruit trees. The new virology lab was one of 16 pioneering research laboratories set up by USDA to define the laws and principles of basic problems in agriculture.

The virology lab was created under the guidance of then administrator of the Crops Research Division of ARS, Marion W. Parker. Several years earlier, Parker had gained much recognition for his scientific spadework with botanist Harry Borthwick toward the eventual discovery of phytochrome, the principal light-responsive pigment in plants, described by Borthwick, Sterling Hendricks, and colleagues at Beltsville in 1959. Parker knew firsthand the value of creating an atmosphere of freedom in a basic research laboratory.

Steere also believed passionately in the need for freedom in basic research and saw Diener as a key person in the new laboratory. He brought in Diener to "look for the nature of symptom expression in virus-infected plants and to develop methods for isolating and purifying plant constituents made by the host in response to virus infection."

"The laboratory was not to be just another TMV lab," says Steere, referring to tobacco mosaic virus. "Everybody seemed to be working on TMV." It had been the first virus purified (by Wendell M. Stanley in 1935) and as one of the easiest to study, essentially became the laboratory rat for the rapidly maturing field of plant virology.

At Beltsville, then, Parker and USDA had opened an opportunity for a very creative atmosphere for plant virus research.

Steere would later say, when Diener received the Alexander von Humboldt award in 1975, "perhaps the most important lesson to be gleaned from the discovery of the viroid is the importance of freedom of research scientists to follow leads when they become evident, rather than be tied down by too narrow a position description and predetermined goals..."

For example, after Raymer first sought Diener's help, it took 6 full years of intense experimentation before they published in Science the first paper on their unusual findings: "Potato Spindle Tuber Virus: A Plant Virus with Properties of a Free Nucleic Acid," which proposed the term "viroid."

Diener (right) and Dennis R. Smith in August 1971. After discovering the naked cellular intruder, a viroid, Diener double-checked all the experimental evidence for 2 years before publishing his results in 1971. Smith has been Diener's top support scientist from 1969 to the present.

Diener, the Concept Breaker

In science, observations of nature form unifying concepts to help us know more about the universe. Among the concept unifiers, though, are the rare concept breakers like Theodor Diener. Diener says that all too often the concepts of science, the accepted dogmas of the day, are powerful deterrents to discovery. "Too many scientists tend to think that everything has to fit into present knowledge, and as a consequence, all of their experiments are variations on a single theme."

Those who know Diener best say that his iconoclastic attitude toward research made possible the viroid discovery.

Diener actually formed a doubt-the-dogma view of science long before he put the parts together on the viroid. "As a student at the Swiss Federal Institute of Technology in Zurich, I was told that nucleic acids were simply structural parts of cells, that they merely kept the all-important protein molecules in their proper three-dimensional configurations. Well, I still remember my excitement to learn of the now famous experiments by Avery, MacLeod, and McCarty that clearly showed in 1944 that DNA, and not protein, is the carrier of genetic information. Then I was shocked that these results were long ignored by the scientific establishment. Professors continued to teach that proteins played the central role as the carriers of inheritance. The old concept had become too powerful to be abandoned because of a few solid facts."

Only after Chase and Hershey's work on bacteriophages—the viruses of bacteria—in 1952 confirmed Avery's conclusions, did the attitude of scientists toward nucleic acids slowly change. Less than 20 years after Chase and Hershey's classic work, Diener made his own discovery, now also considered a classic.

Background: How Do Viroids Work?

Although scientists knew about viruses late in the 19th century, it was not until the mid-1950's that the study of viruses become a major field of science. Virology came to the forefront along with key discoveries on DNA and heredity. In particular, the way in which viruses operate—by hijacking a living cell's genes and replacing them with their own—drew scientific work on genetics and in microbiology together, never to be separated.

Before the 1950's, scientists had no direct means to reveal the nature of a gene, the carrier of heredity. They only knew that chromosomes—the scaffolding for genes in the cell nucleus—contain a lot of the nucleic acid DNA.

Nucleic acids are the molecules of heredity. There are two kinds—deoxyribonucleic acid, or DNA, and ribonucleic acid, or RNA. With the exception of some viruses, all genes—the genetic blueprints of a living organism—are made of DNA. Before a cell divides, its DNA duplicates. Each daughter cell receives a complete complement of DNA molecules identical to that of the parent cell.

As molecules go, DNA molecules are huge. But they're quite stable in their double helix configuration—two molecular strands twisted together like stripes on a barber's pole. Aside from when DNA duplicates, the only time it loses its double helix is when it briefly unzips to dictate the making of new RNA molecules.

RNA molecules are not as stable. They're sort of the slave nucleic acid in a cell's genetic communication system.

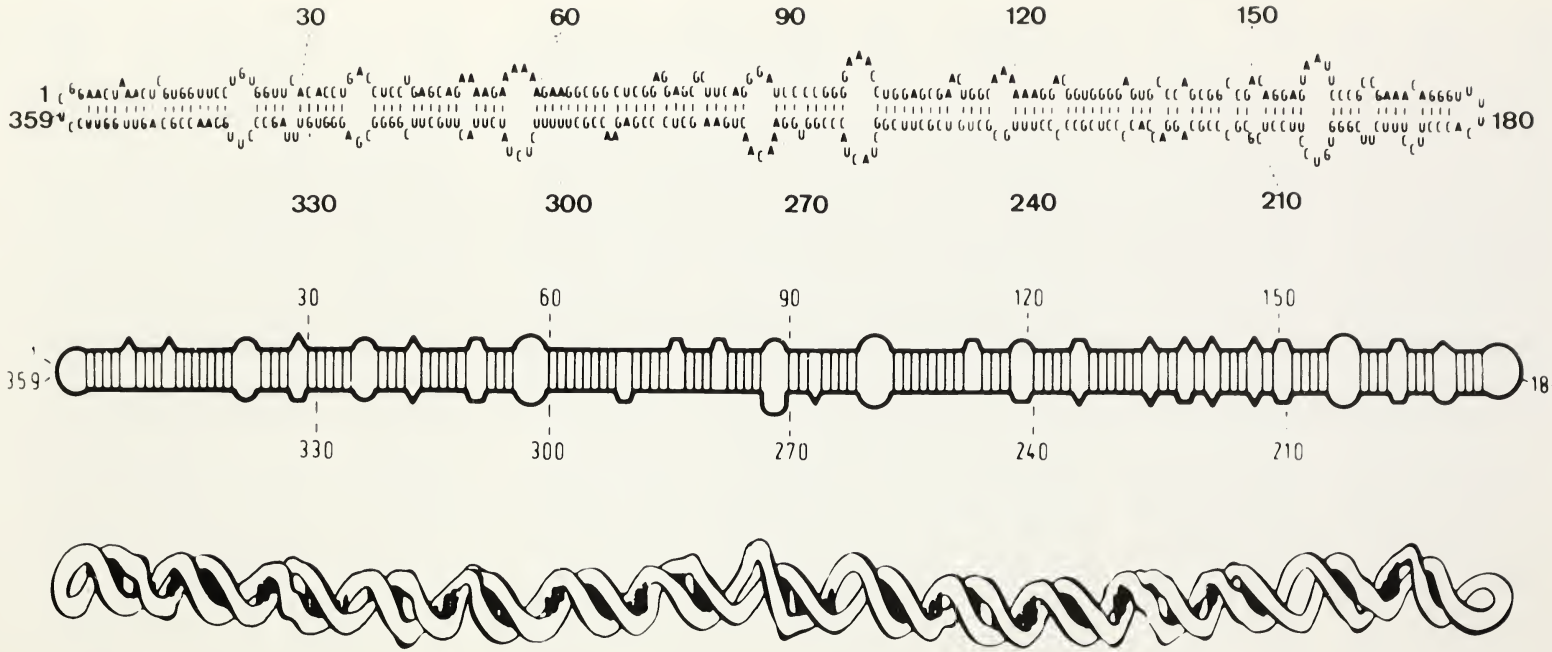
Information on the system is conveyed by linear sequences of DNA and RNA letters called nucleotide bases. Gene messages are written on DNA in the language of four kinds of bases—designated A, G, C, and T—strung like beads on a necklace. Each letter on one strand is delicately linked to a complementary letter on the opposite strand, twisted into a double helix.

When the two DNA strands unzip, lying about are specific RNA nucleotide letters—designated A, G, C, and U—that are brought by an enzyme called RNA polymerase to each of their complementary bases on the DNA strand. The matchups, or base pairings, of letters never varies: The C's of DNA match up with the G's of RNA. The A's of DNA match up with the U's of RNA, the T's with the A's, and the G's with the C's. As DNA strands unzip, their bases form RNA strands with gene messages from DNA. Scientists say that in this way nucleic acids are chemically alive.

The newly formed RNA strands are messengers. They "swim" from the cell nucleus to ribosomes in the cytoplasm or cell soup. Ribosomes are protein factories where the gene messages on the messenger RNA's order the building of amino acids into proteins.

Any living thing, then, is an expression of processes driven by proteins. The proteins are made at the commands of RNA molecules, which in turn owe their existence to DNA—the genes. These rules of biology, developed during the past 30 years, are what the discoverers of the DNA double helix, James Watson and Francis Crick, call the central dogma of molecular biology.

When a virus or viroid enters a cell, its genetic message—the sequence of its base letters—forces the cell to duplicate its viral



Three views of the potato spindle tuber viroid. Above: The nucleotide sequence in a two-dimensional pairing scheme. A loop is created where the genetic letters fail to match properly. Middle: Diagram of PSTV. Below: Loops in nucleotide sequences lead to a likely "twisted rubberband" three-dimensional view.

RNA or DNA instead of the cellular RNA. The cell then perpetuates the genetic information of the intruder at the expense of its own genes.

In this very insidious way, viruses or viroids can be destructive and dangerous to life, in varying degrees of parasitism. Unlike bacteria, which do their damage between and outside of cells, viruses and viroids are always found inside cells—the ultimate parasites.

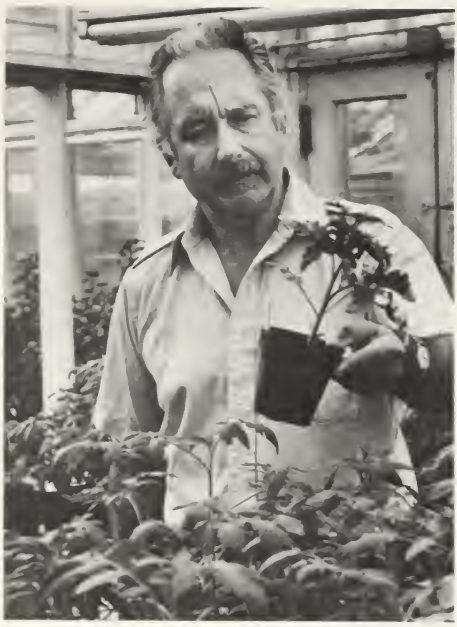
They are such strange agents of destruction that scientists studying viruses in this century have often become fascinated with the question of what life is. On the one hand, viruses are lifelike—genetic systems that reproduce. On the other hand, they are no more than chemicals in a bottle—unless living cells are placed into the same bottle.

Until Diener's discovery of the first viroid in 1971, scientists mistakenly believed that any agent of infection was surely a virus if experiments had failed to produce a bacterium, fungus, or other infectious microbe. They also thought that the smallest viruses that can infect by themselves and self-replicate would need a nucleic acid genetic message with at least a molecular weight of 1 million daltons (a unit of atomic measurement). Anything smaller, so it was thought, could not carry sufficient RNA or DNA to hijack the genetic machinery in a cell, as viruses do. (The only viruses known before 1971 to contain nucleic acids weighing less than 1 million daltons were satellite viruses, but those need a helper virus to replicate.)

The Beltsville Viroid Research Methods

Raymer and Diener, for a year after they teamed up, worked under the notion that the spindle tuber disease agent was a virus with a very unstable structure. They assumed that it fell apart when extracted from tomato plants.

After the puzzling results of the initial centrifuge tests, Raymer and Diener decided to try another technique, called density gradient centrifugation, developed by Brakke. Using this technique, their tomato leaf extracts were spun more slowly, but in columns that separated out molecular ingredients according to their sizes. Larger, heavier molecules went to the bottom of the column; smaller, lighter ones stayed toward the top. After spinning some extract, the scientists dribbled it out of the bottom of the centrifuge column. They tested layer after layer from bottom to top of the column on healthy tomato plants. If the infectious agent had been a virus it would have been in the bottom. But the bottom layers didn't cause the disease.



Diener with some of the 800 tomato seedlings needed each week in viroid "pilot demonstration" at Beltsville.

The upper portions, however, showed some infectivity. "The slow rate of sedimentation of the infectious material made it unlikely that the agent was a conventional viral nucleoprotein," says Diener. "It appeared more likely that this material was a free nucleic acid."

Procedures in enzyme chemistry were next. Diener and Raymer treated extracts of sick tomato leaves with an enzyme that chews up RNA. With RNA removed from the extracts, the scientists discovered that the treated extract failed to reinfect healthy tomato plants as it had before the enzyme treatment. RNA in the agent was clearly important.

Next they treated extracts with an enzyme that chews up DNA, then with another that eats protein. Neither changed the agent's ability to infect tomato plants. The results told Diener and Raymer that the essential ingredient of the spindle tuber agent was RNA and that, much to their surprise, it contained no protein. "We already knew then in 1966 that this was a nucleic acid at very, very low concentrations," says Diener.

The two scientists ran many other experiments that confirmed their hypothesis that the strange disease is caused by a naked bit of RNA. Unlike viruses, the viroid consists solely of the nucleic acid RNA and doesn't have a protein shell. On a virus, the protein shell is that part that surrounds and protects its all-important nucleic acid.

Raymer, who says that he "leans more toward the practical side of research," left the project in 1966 for an industry position.

Gaining Physical Evidence of a Viroid

After Raymer left Beltsville, Diener turned his attention to capturing critical physical evidence of the naked, self-replicating RNA disease agent. An essential first step was to get more precise information on the size of the agent.

Sizing it was difficult. Only miniscule amounts of the infective RNA are present in infected tomato leaves. Because of the very low concentration of the viroid RNA, conventional techniques of determining molecular weight could not be used.

Instead, he used a combination of two techniques for sizing nucleic acids that did not require their isolation in pure form: sedimentation analysis of the centrifugation work already described and gel electrophoresis. In gel electrophoresis, a change of several hundred volts is established across a gel, usually made of polyacrylamide. When applied to the gel, negatively charged molecules such as nucleic acids migrate toward the positive pole. As they move through pores in the gel, the nucleic acids separate according to size. Small nucleic acid molecules move quickly through the pores of the gel; larger ones move more slowly.

Diener could measure the movement of his PSTV (potato spindle tuber viroid) nucleic acid on the gel against standards already established for different kinds of nucleic acids and come up with a relative size of the PSTV.

He applied extracts of PSTV-infected leaves on top of the sausage-shaped gel, subjected the gel to electrophoresis, and then cut it into thin disks. He ground each disk into solution and rubbed it onto leaves of several healthy tomato plants. It was then a simple matter to pinpoint where the infectious RNA had been located on the gel. He found that only plants inoculated with material from near the positive pole of the gel developed symptoms.

The combination of the gel analysis with the sedimentation data showed conclusively that the RNA of the PSTV agent was far smaller than the 1 million daltons that virologists at the time thought was minimally required for infectivity. The gel evidence pointed to an RNA strand of nucleotide letters that according to the central dogma of molecular genetics would only be long enough to code for a cell protein of a molecular weight of about 10,000. That small size, according to virological principles, was not enough for a self-replicating viral agent. With such short RNA strands, the agent would have to depend on the host cell enzymes to replicate itself. Evidence confirming the low molecular weight of viroids was added in the early 1970's, by Heinz Sänger and coworkers at Giessen, Germany.

The small size raised more questions.

For example, was the PSTV one of the satellite viruses that require a helper virus for their own replication? Diener tried in several ways to find a helper virus in uninfected tomato plants but couldn't.

Did the agent consist of more than one type of molecule?

Diener ruled that out by observing the particular way in which its infecting ability diminished when diluted preparations of extracts were rubbed onto test plants.

Diener knew he had something very special at this point: a very tiny bit of disease-causing, self-replicating RNA without a viral protein coat. A naked cellular intruder. "I sat on this work for 2 years to make very sure all the holes in the evidence were plugged before I published it in 1971," Diener recalls.

With a molecular weight initially estimated as 50,000 daltons, but later revised to 130,000, the potato spindle tuber viroid is 80 times smaller than the smallest known virus.

"The high priests of molecular biology," Diener says, "did not believe me at all."

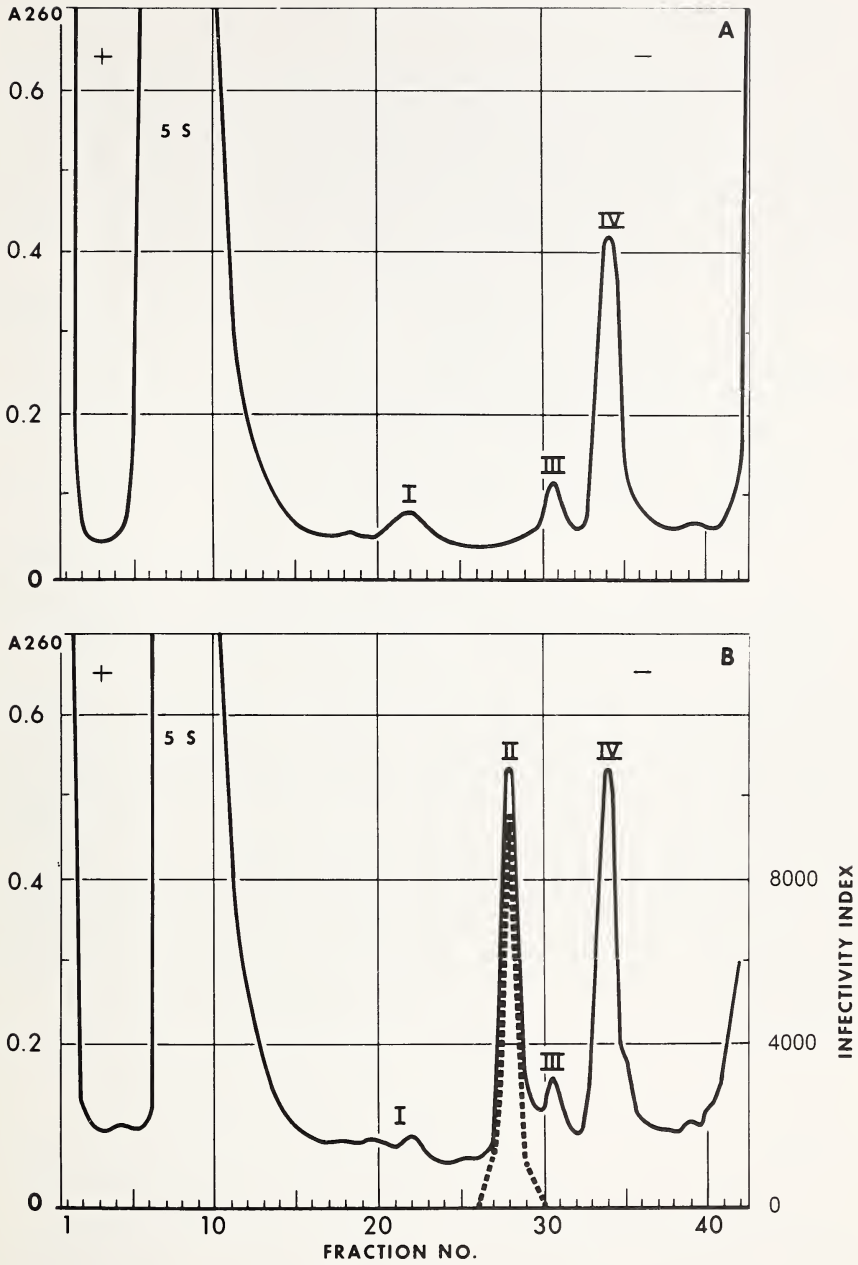
But there was more. Diener had nerve enough to suggest that this very small RNA pathogen copies itself in cells. It was self-replicating, without any protein, he said.

In 1972, Diener published comparative graphs that many virologists now call classics. The two graphs showed distribution of the amounts of ultraviolet light absorbed by the electrophoresis gel where infectivity had been found. One graph was from a gel in which a nucleic acid extract of healthy tomato plants had been electrophoresed. The second graph was from a gel in which an identically prepared extract from PSTV-infected plants had been electrophoresed. The two graphs are the same, except for one thing. On the graph of the infected extract, a sharp peak reveals PSTV. The peak corresponds to the precise location in the gel where PSTV had first shown its infectivity when rubbed onto healthy plants.

The graphs were the first recognition of a viroid as a physical entity.

The physical evidence for the viroid—specific gel disks containing the agent—then made it possible for Diener and others to try to purify the PSTV and get a look at it through a microscope.

Meanwhile, he knew that the long-sought agent for potato spindle tuber disease was drastically different from any viral pathogen so far studied. For the first time in Diener's career, this critic of scientific dogma was defining concepts of molecular biology that others would, in turn, challenge.



First physical evidence of the viroid. Each peak of the graphs represents a small RNA nucleic acid in tomato leaf juice found by gel electrophoresis. The peaks show levels of ultraviolet light absorbed by the RNA's. The horizontal line of the graph shows the relative positions of each RNA on the gel. Graph A is for RNA's from healthy plants. Graph B is for RNA's from plants infected with the potato spindle tuber viroid. Peak II, the viroid RNA, corresponds to the gel position and high infectivity of PSTV (dotted line).

The Skeptics

Looking back on "the contention following my report of the viroid," says Diener, "I remember that most plant virologists accepted it because they were familiar with my previous work. But it was those working in animal virology and in medical research who were the doubters. They weren't used to proving the molecular weight of something by rubbing it onto tomato plants."

Hugh D. Robertson, associate professor in the Laboratory of Genetics at Rockefeller University, says that "even though many now think of Diener's laboratory at Beltsville as sort of a historic site, there were a lot of knowledgeable scientists who didn't believe him at first. There was real skepticism." Robertson studies viroids of plants and viroidlike pathogens of mammals and humans.

He recalls that shortly after Diener's first papers on the PSTV appeared in the journals Virology and Science, he invited Diener to give a lecture at Rockefeller. "In order to drum up an audience for this obscure plant pathologist, I went around to my colleagues to ask them to bring some of their post docs. One of the big shots in RNA metabolism at that time had just arrived here. When I told him that Diener had an RNA that was self-replicating, he said 'Hugh, I bet you a bottle of Scotch, they don't exist.' In 1974, that was the prevailing attitude."

The viroid was indeed a strange thing to accept, especially for animal and human virologists used to dealing with mammalian cells. Karl Maramorosch, professor of entomology and microbiology at Rutgers University, remembers the first time that Diener presented his evidence at a conference of comparative virology at Mt. Gabriel in Quebec, Canada, in 1969. "Unlike others who may have talked or dreamed about a naked RNA pathogen, Diener had documented it experimentally. After Diener's presentation to about 200 people, Nobel laureate André Lwoff (one of the founders of virology) asked him question after question for a very long time. Dr. Diener confidently and very kindly responded to him and proved to everyone's satisfaction, I think, that this viroid was not a nucleic acid from a virus but a new type of subviral pathogen."

Eventually, research by Diener and others to learn how viroids multiply and cause disease showed clearly that viroids could help scientists look into basic biological processes of genes. Because of their small size and simple genetic makeup, viroids are now excellent probes for learning the secrets of genes

that control a plant's susceptibility or resistance to disease. Scientists are using viroids to learn how and at what growth stage those genes turn on and off and how pathogens get around resistance genes to make plants or animals sick.

Meanwhile, as a novel type of pathogen, viroids have alerted scientists around the world to the possibility that other unknown types of disease-causing agents may exist.

Other Viroids Confirmed the Discovery

While Diener was meticulously gathering evidence pointing toward a naked, self-replicating piece of RNA, several other researchers were on the viroid trail. This competition is one part of the viroid story that surprises none of the experts. Science is, after all, much more a social endeavor than it is the work of isolated individuals.

In 1975, Diener was a co-recipient of the Alexander von Humboldt award presented each year for the most significant contribution to agriculture or the agricultural sciences for the past 3 to 5 years. In accepting, Diener said, that "in science...in contrast to many other areas of intellectual endeavor, no scientist stands by himself, but rather on the shoulders of all his predecessors, without whose work his accomplishments would not be possible."

The other recipient of the Von Humboldt award that year was University of California virologist Joseph S. Semancik, for his research on unraveling the nature of another mysterious "viruslike" pathogen, the cause of the citrus exocortis disease.

Semancik published a paper in 1970 in the journal Phytopathology, "Exocortis Virus, an Infectious Free-Nucleic Acid Plant Virus with Unusual Properties." He and coworkers were onto another viroid-caused disease, known only since 1948.

The citrus exocortis disease (from "exo," meaning "outside," and "cortis," meaning "bark-related") causes a peeling or shelling of the bark of certain orange, lime, and other citrus crops. The disease is found in all major citrus-growing areas of the world.

The exocortis disease, similar to potato spindle tuber disease and others later discovered to be associated with viroids, is not spread by insects, as are many plant viruses. Instead, viroid diseases seem to be spread by people and their tools—in cultivating, harvesting, or processing of crops.

Viroid diseases occur mostly in crops that are propagated vegetatively, that is by cuttings, rather than in those grown from seeds. When citrus stem cuttings are grafted onto nursery root

Citrus exocortis disease, caused by a viroid, damages the bark of citrus trees. Left: Normal benching or outgrowth at base of orange tree. Right: Exocortis-viroid-infected tree shows scaling of bark, particularly on trunk bench.

stock before going out to orchards, the workers probably introduce the exocortis viroid into citrus tissue. When potato workers cut pieces of seed potatoes for planting the next crop, their knives contaminate cells of healthy seed potatoes with the PSTV.

Diener has proposed that viroids are prevalent in nature, that there are great viroid reservoirs in wild plants where they go unnoticed. He says that they cause diseases in crops when accidentally transmitted into cultivated potatoes, citrus, or other vegetatively propagated plants. Viroids are opportunists.

Since Diener's discovery of the potato spindle tuber viroid and Semancik's work on citrus exocortis, scientists have found viroids causing diseases in cucumbers, tomatoes, hops, chrysanthemums, and coconuts. Also, dozens of other diseases of plants, animals, and humans show signs of being viroid-incited.

Of viroid diseases known so far, the coconut cadang cadang disease is the most severe, causing the death of about one-half million coconut palm trees per year. Cadang cadang is the main threat to coconut production in the Philippines. It has killed an estimated 20 million palm trees in the past 50 years.

Coconut cadang-cadang disease. Above left: Early viroid infection. Nuts are smaller and more numerous than normal. Fronds appear normal. Above right: Late viroid infection, 1 to 4 years before the tree dies. Nut production has stopped. Fewer fronds remain and have become brittle and appear ragged. Below: Coconuts from healthy (left) and viroid-infected palms.

Viroid Findings Open New Avenues

All viroid investigations until the late 1970's had been in plants. Diener, however, observed in his book, "Viroids and Viroid Diseases," "In view of the uniform biochemical basis of all life on earth [referring to the DNA-RNA-protein genetic code, consistent from bacteria to man], it is difficult to understand why viroid-like nucleic acids should exist only in higher plants....The etiological agents of one group of animal diseases, however, possess certain properties suggesting similarity with plant viroids." In 1977, this group was named "slow virus infections caused by unconventional viruses," by neurologist D. Carlton Gajdusek, who investigates human neurological diseases at the National Institutes of Health. The slow viruses include scrapie of sheep and goats, mink encephalopathy, and Kuru and Creutzfeldt-Jakob disease in humans. In all cases, the agents cause brain lesions and invariably lead to death.

Reminiscent of William Raymer's rabbits that failed to produce antiserum to the potato viroid in 1961, none of these diseases stirs the victim's immune system to fight back. Therefore, the agents might be similar to viroids in lacking a protein coat that would be detected in laboratory analysis.

In fact, commenting on Diener's curiosity, Raymer says that after Diener published on the potato viroid, "he was trying very hard to get the animal scientists to look for viroids with the same intensity that he did with the plants."

Diener took particular interest in the scrapie agent. Clear evidence from other labs pointed to a very small size. Diener wrote that his potato viroid made it "apparent that the small target volume of the scrapie agent did not necessarily rule out nucleic acid as the genetic material."

In 1972, Diener put out the word. He published a paper in Nature, "Is the Scrapie Agent a Viroid?," in which he spelled out the evidence and challenged others to find it.

Several years later, Diener and Stanley B. Prusiner, professor of neurology at the University of California, San Francisco, ran experiments aimed at finding small pieces of nucleic acid in tissue from scrapie-diseased sheep.

In 1981, Prusiner discovered that the scrapie agent did have a protein after all and that it needs the protein in order to infect tissue. On the other hand, the scientists haven't found that the agent needs a nucleic acid to be infectious. Therefore, it couldn't be a viroid. And they found that the scrapie agent is even smaller than viroids.

To Prusiner, all of this strange evidence meant that—just as Diener 10 years earlier had presented the world with a prototype of a new kind of pathogen—he too would break with convention. Prusiner announced a second class of subviral pathogens. He called the scrapie agent a prion.

In enzyme analysis, prions proved to be resistant to deactivation by most laboratory procedures that alter nucleic acids. It's still not fully clear if they require any nucleic acids at all, their own or those from host cells, to be infectious.

But one thing was clear to Prusiner. He could take solace and comfort, he said, in the way that Diener had dealt with criticism of his viroid. "All the difficulties he experienced we are still going through with the prion investigations. There are relatively few scientists who are as open minded as Ted, yet he is very precise, very critical, and very, very careful. Those qualities allowed him to make the first viroid discovery, which made feasible the idea of very small infectious nucleic acids.

"Now, the prion story is even more radical than the viroid as we now know it, yet Ted had no problem dealing with the prion problem either."

While there have been no human cases of scrapie, the investigations of prions serve medical science as an excellent model for the study of a wide range of brain-degenerative diseases, such as Alzheimer's, says Prusiner. All of the work which has been done on the scrapie prion applies as well to studies of the human prion disorder, Creutzfeldt-Jacob disease, which was first transmitted in experimental animals by NIH's Gajdusek.

Will viroids be found in humans?

"That's actually not the important thing about viroids," says Gajdusek. "The important thing is that they've made us look. I get very upset when people decide that if we find viroids as human pathogens, that is when they will become important. No, that's not it! Agriculture is 10 times more important to human health. Health depends first on having enough food from agriculture before you can even start talking about stopping human pathogens [in medical research]."

Gajdusek received a Nobel Prize in 1976 for discovering that Kuru, a neurological disease of certain New Guinea tribes, is infectious and transmissible to chimpanzees. He says that Diener, as a contributor to scientific progress, deserves more credit than many more famous scientists. Diener's initial investigations leading to identification of the first viroid, says Gajdusek, add up to a rare scientific "pilot demonstration," in which "he was a very hard plodding prover of the work as well as the discoverer. Diener's contributions in working out all the details

were enormous, and Beltsville deserves tremendous credit for helping him to do things right."

According to Rockefeller's Robertson, viroids will be found to cause human diseases, but "only after virologists gain a better idea of the kind of RNA to look for." Says Robertson, "All RNA metabolism in higher organisms, plant or animal, is extremely complex, and each one different. Therefore, we are going to have to work very hard to understand the unique viroid environment for each life form. In stalking a simple viroid, we are looking for the needle in the haystack." The human disease that seems to be most like a viroid infection today, he says, is delta hepatitis, a form of hepatitis that is apparently caused by a subviral agent working in conjunction with the hepatitis B virus. Diener agrees that there are some nucleotide-base-sequence similarities.

Also, viroidlike RNA's have been found to be associated with human Crohn's disease by a team of German medical researchers (Reinhart Pechan, Hans Kunert, and Hans J. Gross). Crohn's is a condition that causes the lower digestive tract to leak and malfunction. President Dwight D. Eisenhower suffered from Crohn's disease. Evidence shows that a viroidlike RNA is found whenever tissue is examined from the intestines of a Crohn's patient.

Mum Stunt, Diener's Second Viroid

As Robertson suggests, knowing what to look for is the key to tracking and learning about any small pathogen. Whereas it took 6 years for Diener and colleagues to find the first viroid, he needed only 90 days to find the second.

Chrysanthemum stunt disease was brought to Diener's attention by ARS horticulturist Roger H. Lawson, now research leader of the ARS Florist and Nursery Crops Laboratory at Beltsville.

"When Ted published his potato spindle tuber viroid paper, I told him that the agent for chrysanthemum stunt shared some of the same analytical traits." Extracts of chrysanthemum leaves with the disease showed high levels of infectivity from the upper portion, or supernatant, of a centrifuge, as the potato disease had. Heating the extracts would change the infectivity, but the agent was easily transmissible from plant to plant in tests, the same as PSTV. "It looked like very similar evidence," said Lawson.

Diener and Lawson repeated the PSTV experiments with extracts from chrysanthemum leaves. They found a viroid that was distinct from the potato spindle tuber viroid.

Chrysanthemum stunt disease, caused by a viroid, spots leaves and turns them yellow. The disease was once an epidemic in both the United States and the United Kingdom.

The agricultural circumstances were very different. Whereas potato spindle tuber was more of a scary disease to potato breeders than a serious problem to farmers, chrysanthemum stunt disease had once caused a full-blown epidemic.

"It nearly devastated the mum industry both here and in England in the 1940's," says Lawson. "It was so severe that in some large greenhouse nurseries, as you viewed benches of thousands of plants, it was the occasional healthy ones that stood out."

The problem with commercial mums and the viroid was that the plants are propagated by hand. Billions of stem cuttings are propagated in an industry that is highly concentrated in just a few large commercial companies. Plant pathologist A.W. Dimock of Cornell University first described the disease in 1947 and showed that it can be spread quickly by propagators' knives through nursery stock.

In the 1940's, Yoder Brothers, Inc., today the leading chrysanthemum producer in the world, developed a diagnostic test. It was a laborious method of hand-grafting and waiting that took 6 months to a year to eliminate stunt from propagation stock.

In contrast, discovery of the stunt viroid has led to a 2-day diagnostic test based on technology developed in Diener's laboratory. The test helps propagators avoid disease transmission. Today, stunt is only a minor annoyance in an occasional propagation greenhouse, and sales of potted and cut mums in the United States are $225 million annually.

Further Research To Purify Viroids

After the pilot demonstration stage, Diener's team at Beltsville as well as other viroid researchers turned to finding out more about how viroids operate. They needed to purify PSTV. Methods used at the time to purify viruses were useless because there were no true virus particles to capture.

Instead, the strategy was based on getting large amounts of highly concentrated nucleic acid extracts from tomato plants and laboriously removing cellular RNA from the viroid. For their experiments, Diener's team was using up to 800 tomato seedlings per week to extract and purify PSTV. Dennis R. Smith, Diener's top assistant since 1969, recalls that over 500 tomato plants were needed to get 2 kilograms of tomato leaf juice which, in turn, yielded 1 milligram of concentrated PSTV. There were a couple of acres of potted tomato plants under glass for viroid experiments. Smith and Diener would apply the concentrate to gel strips. After electrophoresis, they would stain the strips. Narrow distinctive lines marked the presence of the viroid. They cut out the narrow markings on the gel, removed the viroid from the gel, reconcentrated it, and ran other cycles of electrophoresis to further purify it.

At this point, the team wanted to find out if the PSTV was single- or double-stranded RNA, in order to learn how the RNA multiplies, or replicates, in living cells. Such information would be helpful in designing strategies for controlling viroid diseases of crops.

They used a technique called heat denaturation. Scientists have learned through experiments that when heat is applied to a nucleic acid, its base-pairing melts or breaks apart. The temperature at which a nucleic acid melts, or denatures, is determined by an instrument that can read how much ultraviolet light it absorbs as heat is increased.

Standards had already been set for how much ultraviolet light is absorbed by different nucleic acids. Diener's team knew, for example, that double-stranded nucleic acids absorb less than single-stranded ones.

However, the denaturing experiments produced one more mystery about the viroid. The scientists discovered that it fell somewhere between standards for single- and double-stranded RNA in absorbing ultraviolet light while being heated. Did this mean that the viroids were partly double stranded? There was no precedent for that!

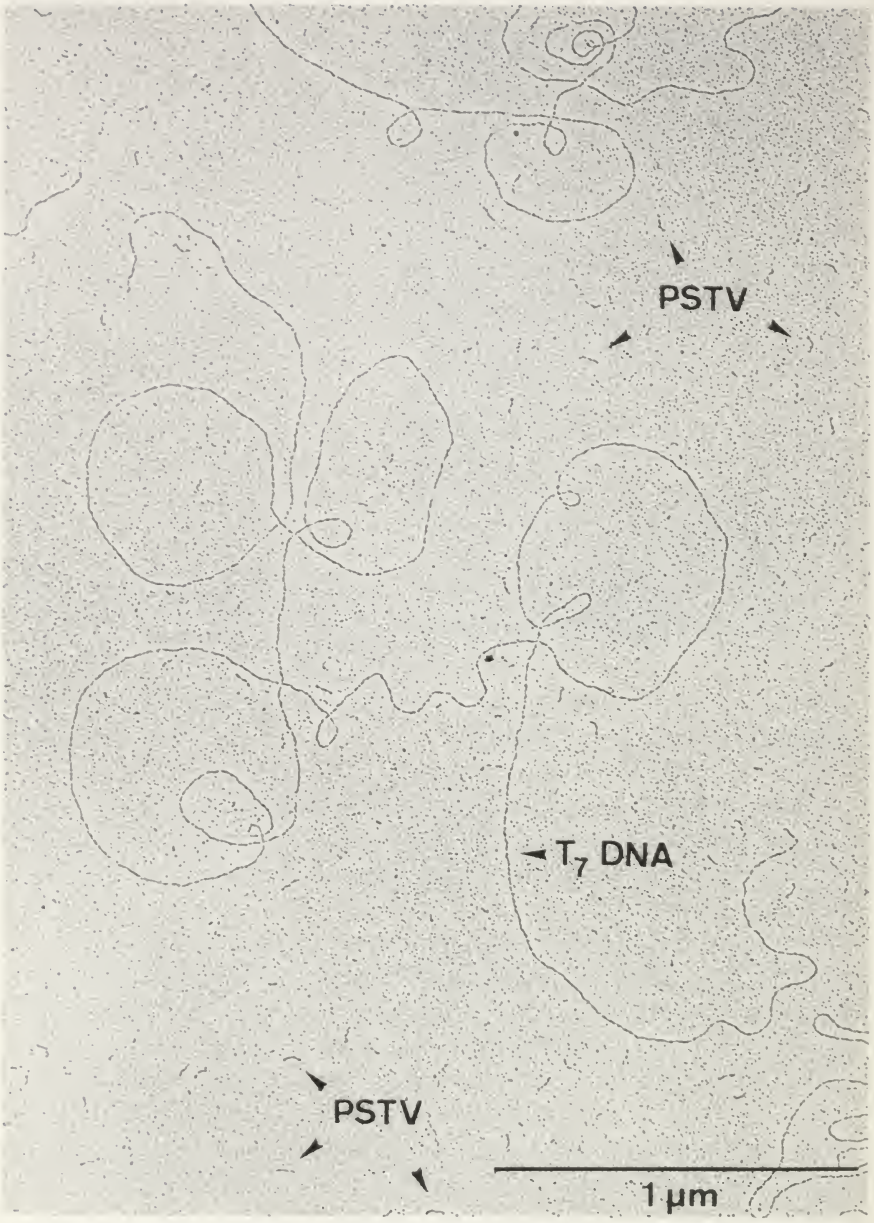
Scientists isolated the potato spindle tuber viroid from narrow lines on an electrophoresis gel.

Getting a Look at a Viroid

Meanwhile, purer samples of PSTV and other viroids became available in the late 1970's. This meant that Diener's team, as well as teams of scientists elsewhere, could try to get a look at a viroid.

Viroids and viruses are smaller than one wavelength of visible light. They cannot be seen by an ordinary light microscope, which magnifies only about 1,000 times. Electron microscopes use a beam of electrons whose wavelengths are smaller than viroids. An electron microscope can magnify an object several hundreds of thousands of times. Through this instrument, scientists can see and measure widths and lengths of DNA or RNA that are coiled or strung into a virus particle, or, as it turned out, in a viroid.

Diener gives credit to a young postdoctoral researcher from Spain, José Sogo, for leading the way to the first pictures of a viroid, PSTV. It happened while Diener was visiting Professor Theo Koller at the Institute of Cell Biology at the Swiss Federal Institute of Technology in Zurich. Sogo tried an adaptation of the Kleinschmidt method, the first technique invented to prepare nucleic acids for being seen with an electron microscope.



The width or thickness of potato spindle tuber viroid is seen in a micrograph. A DNA called T7 is also seen beside the much smaller, rod-shaped PSTV.

The group of scientists added a compound called cytochrome C to a preparation of purified PSTV. The cytochrome C causes the liquid preparation to spread into an ultrathin layer, one molecule deep, on top of very clean water. It's the same sort of thing as an oil slick on a puddle.

The resulting pictures, called electron micrographs, revealed PSTV as uniform rods with an average length of 50 nanometers. (One nanometer is equal to a billionth of a meter.)

The micrographs confirmed irrefutably the very small size of the "scare-factor" disease agent that had eluded plant pathologists for over a half century.

What about width or thickness of the rods, an indication of the RNA's strandedness? The scientists had prepared their sample of PSTV mixed with DNA molecules of a bacterial virus called T7. The size and shape of the double-stranded T7 DNA were already well known. T7 led to another viroid mystery, but a short-lived one this time.

In the micrographs, the PSTV rods appeared to be the same thickness as the double-stranded DNA. However, the scientists already knew from other experiments that PSTV couldn't be composed of a genuine double-stranded RNA.

Therefore, Sogo, Koller, and Diener suggested that the viroid was a hairpin-shaped structure, held together by extensive but not complete base-pairing of nucleotides between the folded-over sides of the molecule. They published the hypothesis that viroids are indeed single-stranded molecules, but that they fold into what appears to be a double strand.

In 1975, W. McClements and P. Kaesberg at the University of Wisconsin at Madison, and later Heinz Sänger and coworkers in Munich, West Germany, took the first electron micrographs of completely denatured viroid molecules, in which denaturation had broken the hairpin base-pairing. In both sets of pictures, for the first time, two kinds of viroid structures appeared: Straight strands about twice as long as undenatured viroids, and, to everyone's surprise, says Diener, there were also circular viroids. The circles had a circumference also of about twice the length of viroids seen previously.

The pictures explained the results of Diener's denaturing experiment in which PSTV seemed to be halfway between single- and double-stranded molecules. "We already knew, because of the base-pairing, the linear RNA molecule would be folded over itself in a sort of hairpin structure," says Diener. "Sure enough, when denatured, the molecules opened up twice as long as before."

The discovery on electron micrographs that viroids have at least two forms, rods and circles, helped scientists understand how they may replicate or multiply in cells.

More importantly, the discovery of the circles underlined the unique characteristics of viroids. Such a circular nucleic acid had never been found before. They are covalently closed circles of RNA, meaning that they are completely linked by strong chemical bonds.

Viroid scientists later proposed that the circles help viroids replicate. A rolling-circle mechanism, proposed first by Andrea Branch and Robertson at Rockefeller University, is the accepted theory. Scientists think that a viroid is copied by special use of a host-cell enzyme.

The evidence is straightforward: Viroid-sick plants not only have full-length viroid complementary strands, but also contain viroid molecules that are much longer than the standard PSTV length. They are presumably formed when the host enzyme starts copying nucleotide bases at one point on a viroid circle, then continues copying beyond its original starting point—thus forming molecules that are several times longer than a viroid.

Earlier experiments showed that during their replication, viroids don't become substitutes for messenger RNA's in the cell. In other words, the viroids don't code for proteins, as might be expected. Scientists have shown that the viroid RNA doesn't interfere with the translating process of genuine messenger RNA. Comparing proteins found in healthy plants to those in viroid-infected plants reveals no new proteins in the sick plants.

When the circles were first seen in 1975, viroid investigators wondered which type of viroid was the infectious type, the circles or the rods. Biochemist Robert A. Owens joined Diener's team in 1975 and quickly devised a laboratory procedure to separate the circles from the rods by denaturing gel electrophoresis.

When preparations of each were rubbed onto healthy tomato plants, the scientists learned that both types of RNA were infectious.

Before Owens' procedure, "It had been a big bone of contention in our field as to which form was the native viroid," recalls Diener. "We used to go to meetings and have big arguments. Some people would say that the linear molecules are just artifacts of the purifying procedures, that laboratory handling broke up the circles. We said no, they're infectious too. Eventually, we learned some do get nicked during the purification, but native linear viroids exist too."

In recent years, rapid methods for mapping the actual sequences of nucleotide letters of RNA molecules have helped scientists to know more about their structures and to sort out what the different viroid shapes mean.

The first to determine the complete sequence of a viroid, that of PSTV, was Hans Gross in Germany in 1978. Gross and coworkers showed that PSTV molecules are each made up of 359 nucleotides. As expected, because of the odd, hairpin shape, they also found extensive, but incomplete base-pairing of the nucleotides. Diener calls it quasi-double-stranded. The nucleotide bases on the hairpin don't always match up with their natural complementary base—the A bases don't match up with their natural complement, U bases, and C bases don't always meet their complementary G bases. The result is a lot of loops in the RNA strands that hang out of line.

An artist's conception of the structure would be sort of a sloppy double helix or a twisted rubberband.

Owens Takes the Team Hybridizing

By all accounts, viroid investigations in Diener's laboratory benefited considerably from the arrival of Owens. His job was to provide ideas that might help the team to more closely study viroid replication and how the tiny pathogens cause disease.

Owens said that before arriving at Beltsville, he knew nothing of the prominent ARS science center, "except what I read in textbooks about Borthwick and Hendrick's discovery of phytochrome." But it was Diener's "scientific instincts that

came through to me. My first impressions of Ted have not changed at all—his remarkable breadth of knowledge of biology and pathology. With these principles as his guiding star, Ted is not wedded to any particular technology or experimental approach. He sets his compass, so to speak, by biological principles. More than anything else, opportunity to work with Ted is why I am happy to be here at Beltsville."

Owens recalls that "it was great" to get back into plant virology, the field in which he had earned his Ph.D. at the University of California, Davis. At Beltsville, he would concentrate on using nucleic acid hybridization. The technique allowed for the first time the matchup of complementary nucleic acids from different origins in a test tube.

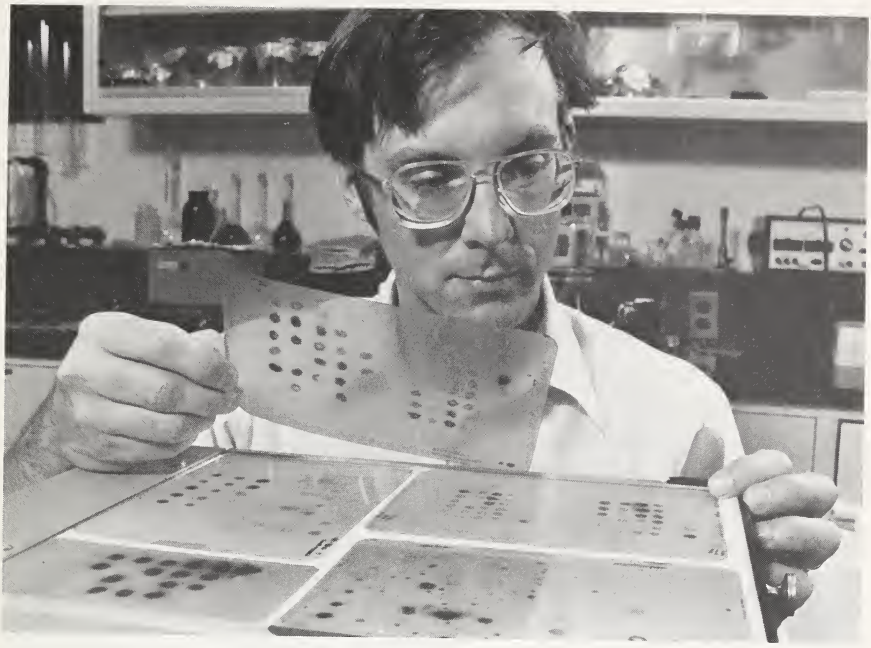
This critical technique got a boost from the simultaneous discovery by H.M. Temin and David Baltimore that certain viruses copy their RNA into DNA using an enzyme called reverse transcriptase. The enzyme transcribes DNA from RNA rather than making RNA from DNA, which had been believed to be the only pathway of nucleotide copying.

Owens, arriving at Beltsville soon after these key discoveries, wanted to put reverse transcriptase to work in viroid research. "It was very clear at that time that nucleic acid hybridization would be very important to the study of viroid replication and pathogenicity," says Owens. "What needed to be done was to use reverse transcriptase enzyme to produce a DNA copy of potato spindle tuber viroid."

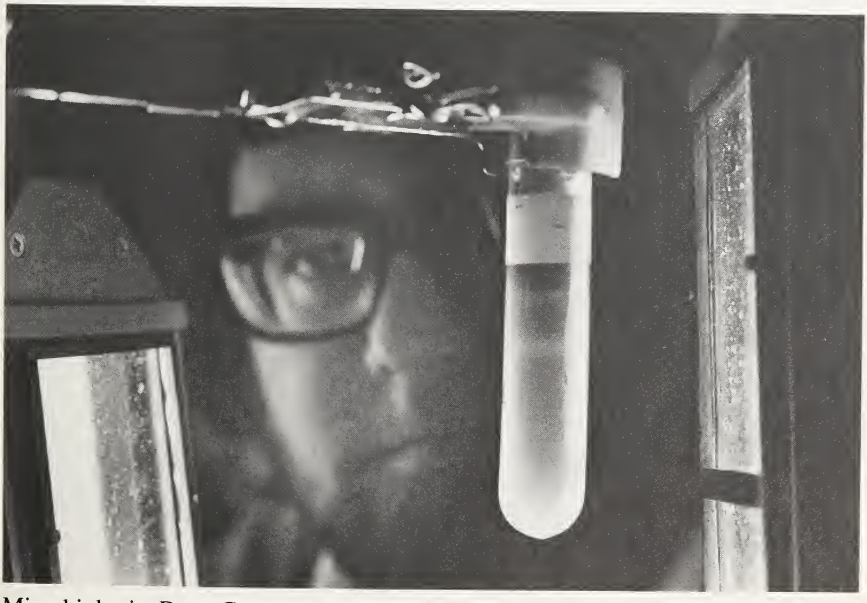
To hybridize a copy of PSTV, Owens would make a mixture of the viroid RNA and short bits of DNA called primers. Then, he would add the reverse transcriptase and the necessary chemical components that induce DNA synthesis. The drawback was that this laborious, time-consuming synthesis of a new batch of PSTV-complementary DNA was required for each separate experiment.

In 1979, just at the right time says Owens, a young microbiologist, Dean Cress, arrived at Beltsville. Cress brought to the viroid project the ability to take the DNA copies of the PSTV—that Owens had been making batch-by-batch—and clone them in bacterial cultures.

Cress was eager to use his expertise in cloning by bacterial plasmids, small circles of DNA in some bacteria. Plasmids are molecular geneticists' main tool for inserting new genes into microbes or plants. "Once he inserted the DNA copy of PSTV into the bacterial plasmid, we could grow an unlimited supply of PSTV-specific DNA" in bacterial cultures, says Owens.



Robert Owens examines developed film of potato spindle tuber viroid used to develop a practical screening test for the viroid. Owens joined Diener's viroid team in 1975 and focused on nucleic acid hybridization.



Microbiologist Dean Cress used recombinant DNA technique to clone viroid copies.

Cloning the PSTV copies in bacterial cultures also improved the DNA copies. Previously, making the DNA copies by hybridization led to contamination, because in the test tube, the hybridizing primer did not necessarily start copying with the first PSTV nucleotide and end with the last. Instead of getting a consistent product, the scientists got a variable population of different combinations. The team then sorted through the population to find and purify the DNA copy that was close to full-sized PSTV.

By using cloning, Owens and Cress could be sure that the DNA molecules matched the viroid RNA exactly. They would know right away that they were working with the potato viroid-DNA copy.

Once the scientists could clone viroid copies, they ran with them in two directions at once. As often happens in science, a bit of laboratory technology, in this case cloning, opens new high-speed roads to further research. In other words, cloning brought viroid investigators to a fork in the road. In one direction, the potential for pioneering work in biology moved into a faster lane. The other fork in the viroid road led to practical screening tests to control the strange pathogens.

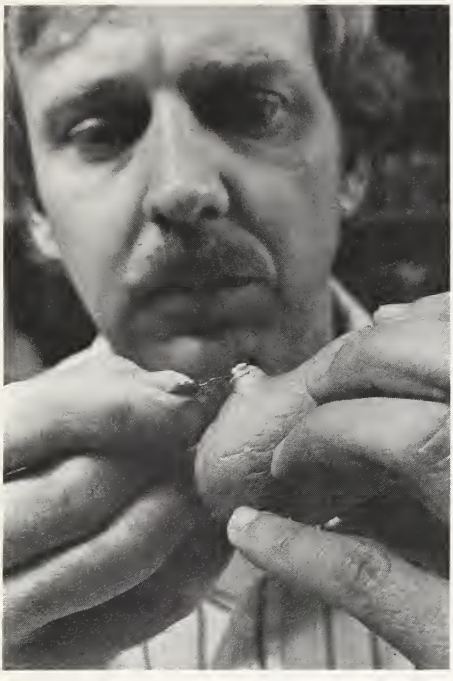
Viroid Control Through Recombinant DNA

Movement down the practical road began in earnest in 1980, when Diener was invited to a planning conference of the International Potato Center (CIP) in Peru.

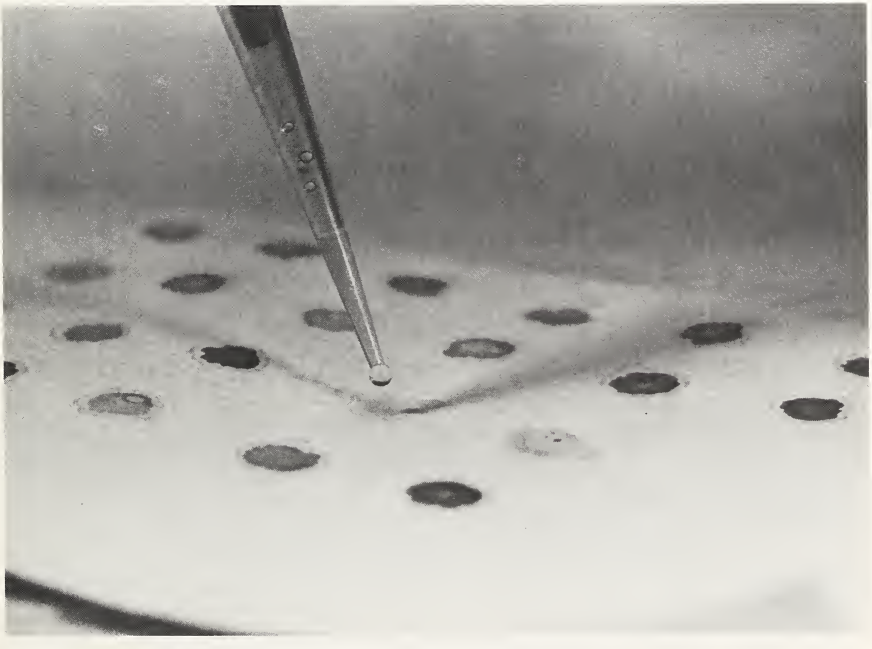
CIP is one of 13 research centers of the Consultative Group on International Agricultural Research. The CGIAR, with an internationally funded budget of $240 million a year on research in developing nations, recognizes that the potato has the potential for producing more well-balanced protein and calories per acre, time, and water than any other major food crop.

When Diener visited, CIP scientists were quite anxious about PSTV because it thrives in warm weather and fast-growing plants. Although potato plants do best in cool weather, CIP is breeding plants for warmer climates. The serological tests that CIP was using to screen their breeding potatoes for viruses would not detect the PSTV. Instead, 3 laboratory technicians ran gel electrophoresis on about 50 samples a week from preparations of extracts of potato plants.

When Diener returned to Beltsville, he said to Owens, "Can't we do something about their problem? Let's see if the hybridization techniques and clones that you're using to study PSTV can help us develop a diagnostic test."



Smith takes sample of potato tissue for a test of the Beltsville technique for rapid screening for viroids.



The diagnostic or screening test for potato spindle tuber viroid uses a radioactive DNA clone of PSTV. Potato plant sap is placed on a sensitive membrane, which is then immersed into a solution of the radioactive probe. If the spot appears dark on a photograph of the membrane, the viroid is present.

Owens and Diener realized after the first experiment that it would work. A few weeks later they wrote a paper that appeared in Science, "Sensitive and Rapid Diagnosis of Potato Spindle Tuber Viroid Disease by Nucleic Acid Hybridization."

Their technique uses cloned, radioactive DNA copies of the PSTV RNA. Potato plant sap is spotted onto a sensitive membrane, which is then immersed into a solution of the radioactive probe. If the spot appears dark on a photograph of the membrane, the viroid is present.

Within 2 years, most potato breeders in the United States were using the technique, according to Chett Sutula, president of AGDIA, Inc. His company markets a kit for testing up to 100 samples of potato tissue for PSTV at once, based on the Owens-Diener technique. Nearly all potato breeders and seed certification programs in the world, including those at CIP, use variations of the new technology.

After market success with the PSTV kit, Sutula asked Owens if the kit might also screen for chrysanthemum stunt viroid. His answer was no. However, Thierry Candresse, a French postdoctoral associate working with Diener, had prepared complementary DNA clones of the chrysanthemum viroid. The clones enabled Sutula to develop a kit to screen for the chrysanthemum stunt viroid.

Diener's laboratory led the way for other scientists to develop molecular probes to detect the cadang cadang viroid. In the Philippines, agriculture officials routinely use the probes to assay leaves of coconut palms in areas susceptible to the disease. The assaying prevents its spread. When a diseased tree tests positive, it is destroyed.

Diener uses viroid clones to study the distribution of viroids in nature. The clones have helped him verify and expand his theory that great viroid reservoirs exist in weeds, with the potential to infest cultivated plants.

Support for the theory recently came from Mexico. Tomato plants infected with a mysterious disease were often found at the edges of plantations near wild vegetation. The disease, now called tomato planta macho disease, causes plants to become infertile. At Colegio de Postgraduados at Chapingo, Jorge Galindo, a professor of plant virology who had worked in Diener's laboratory for a year, became suspicious that the disease was caused by a subviral pathogen. He sent a student to collect weeds near infected tomato plants. The student returned with four wild relatives of tomato plants. All four carried the agent for the disease, which Diener, Smith, and Galindo later identified as a viroid.

Owens hopes that related hybridization and cloning techniques can be used to help relax restraints on the importation of new types of plants by U.S. scientists. Currently, years may pass before plants are cleared through quarantine. In the meantime, the research opportunity is reduced. The plants may even die before they are released for research.

Probing Secrets of Life To Improve Agriculture

The other research road opened by cloning viroids is in further pioneering of basic biology.

Virologists have a strong track record of using viral genetic material, especially RNA, to get at biological secrets of host cells. As a learning tool, there is nothing that is more of a cutting edge in molecular biology, according to Diener, than tinkering with viroids. Usually the cautious investigator, Diener says, "We have with the viroid the most fantastic investigative system in the world today. Using it as a probe into life processes goes way beyond plant pathology. These are very fundamental biological questions which we are now facing."

The development of DNA cloning technique promises tremendous advances because it enables scientists to alter a viroid's nucleotide bases by making slight changes in the DNA copies. The chance to use viroids as probes stems from the opportunity to monitor such mutations in living plants. Because a viroid is small and its nucleic acid, or genetic makeup, is so simple, the mutations are far easier to monitor than similar changes in viruses.

Studies of DNA copies of viroids are being pursued in all the major labs involved in viroid sleuthing today.

On Diener's team, Rosemarie W. Hammond, ARS molecular biologist, is making slight mutations of cloned PSTV. She says the mutants "show us a real association of the subtlety involved between the structure of the viroid and its biological properties."

The team injects one of Hammond's mutated DNA copies into a plant and by means not yet fully understood, the DNA produces viroid RNA that spreads from cell to cell as a native viroid would. While most of the mutated viroid DNA strands are not infectious, some are. In fact, some of those that are infectious show intriguing changes in their patterns of infection. One mutant, for example, affects the viroid's ability to move within the plant, traveling down the stem of a tomato plant to the roots but not back up, as PSTV would.

Meanwhile, Detlev Riesner in Germany recently used RNA sedimentation analysis to show that the enzyme RNA polymerase binds to the viroid in the exact location where two of Hammond's nucleotide changes have altered the viroid's infectivity. In a normal cell nucleus, RNA polymerase controls transcription of RNA strands from DNA. Could it be that some of Hammond's mutations prevent RNA polymerase from binding to the altered viroid molecules and thereby block viroid replication and its ability to infect?

"The seductive thing about this work is that we now have a large number of very sophisticated techniques, many based on recombinant DNA, that enable us to examine the viroid's ability to interact with biochemical mechanisms of a normal cell. Given the fact that a viroid is not encapsulated in an organized protein shell, and it appears that it does not code for any viroid-specified protein, the interaction that causes disease almost certainly involves the viroid RNA itself," says Owens.

Robertson says that if viroid scientists probe around long enough they may find "binding sites" for developmental proteins on the viroid. If so, "viroid studies will profoundly affect our capabilities to turn genes on and off." (Scientists already know that viroid disease symptoms are disruptions in growth and development of the cells. The viroids may be causing imbalances in growth hormones.)

Developmental proteins mentioned by Robertson are important in the eyes of genetic engineers. They aren't the day-to-day housekeeping proteins produced to maintain cellular life. They are instead produced by genes involved with specific stages of a plant's growth and development.

Identifying and engineering specific developmental genes can help scientists produce larger leaves for spinach or kale, for example, or larger or multiple ears of corn per stalk, more protein per wheat grain, or more yams per vine. Developmental genes could be exploited for enhanced photosynthesis, improved nutrition, or faster growth of the food parts of crop plants; the possibilities are endless.

"I think everybody in the field of viroids agrees that viroids are going to tell us many more important things," says Robertson. "There is a universal recognition that once we find out how host cells interact with viroids, we're going to know a lot more about gene regulation and development. It's intriguing that a viroid causes a plant to make a whole series of wrong decisions at the wrong time. Perhaps the viroid preempts a yet-unknown role of cellular RNA."

Says Diener, perhaps indeed the most mysterious thing about viroids is why tomato plants, coconut palms, and avocado trees are so receptive to them. Why is the viroid so much at home in their cells? Cells seem to want to copy them—as if the cell is recognizing a long-lost friend. Yet viroids can't be very good for cells.

Although plant cells are often trapped into helping viruses, usually the crime is committed by interrupting the translation of cellular messenger RNA's. The virus' message jams the cell's gene information at that point and gets the cell's enzymes to make more viral messages. Viroids, however, accomplish the same result in another, not yet fully explained, way.

If the small, naked RNA pathogens—punched into a cell by the climbing cleats of a coconut harvester or the knife of a potato propagator—have no helper virus to hijack a host cell's genes, what machinery of a cell does it take over? Why do viroids incite severe diseases in some plants, whereas in many other plants the same kind of viroid multiplies undercover without any symptoms at all?

The answers could profoundly affect our understanding of life processes. Perhaps at some level in each cell, there is an RNA doing something positive that the viroid preempts. Something we're not yet aware of. Scientists are already aware of elaborate genetic machinery. Why is there a mysterious pathway so readily activated the minute PSTV stumbles into a cell? Such a mysterious pathway probably doesn't exist just for the convenience of the viroid. Does it have some other function? If so, it means that scientists are still looking for the host analog of the viroid that allows the host to be so well prepared for the viroid arrival.

The large number of unanswered questions about viroids is a tribute to the men and women who, since Diener's discovery in 1971, have expertly explored an uncharted area of biology. This story is truly one of scientific curiosity.

Today, viroids as biological probes hold great promise. They can help scientists unravel mysteries of how viruses and subviral pathogens infect plants, animals, and humans. They can point to how growth and development occur in living things.

New biological knowledge, pragmatic crop applications, and promise for further insights have all grown out of a bit of scientific curiosity. It is something that Diener calls "adult play." Scientific curiosity, he says, "is exactly the same instinct as when a child wonders why a beetle on the sidewalk keeps walking in the same direction, regardless of which way it is turned."

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